

# Nitrate Test Kit - Low Range

## MICROPLATE DETERMINATION FOR 96 NITRATE TESTS

This nitrate test kit is based on the enzyme Nitrate Reductase (NaR), catalyzing the reduction of nitrate to nitrite using the natural electron donor NADH. The nitrite reacts with color reagents (dyes) under acidic conditions to produce a visible color. All reaction steps occur in the microplate. The concentration of nitrate in the unknown sample is determined by measuring absorbance with a microplate reader using a 540 nm filter (540 +/- 20 nm OK), and comparing the absorbance to nitrate standard curve.

Nitrate (and Nitrite) can be determined in serum, urine, saliva, plasma, tissue culture media, and extracts of whole organisms; as well as water samples and extracts of plant tissues, soils and foods.

The test is designed to measure nitrate in the range of 3.6 to 71.4 µM nitrate or 0.05 to 1.0 ppm nitrate-N using a 96-well microplate. Nitrite can also be determined by omitting NaR from the test (see Notes on the Tests, page 4).

**IMPORTANT** - Keep contents refrigerated until day of use. See box for expiration date.

### Supplied in NECI Nitrate Test Kit

- Assay Buffer in liquid form - (1) 15 ml amber bottle
- Color Reagent 1 in solid form - (1) 15 ml amber bottle
- Color Reagent 2 in solid form - (1) 15 ml amber bottle
- NADH - 1 tube in amber bag
- Nitrate Reductase (NaR) - 1 tube in amber bag
- Nitrate Standard (100 ppm nitrate-N) in liquid form - 1 tube
- Quench Agent - 1 tube in amber bag
- Microplate - (1) 96-well microplate with flat bottom wells.
- Pipetter Reservoir Basins (2) clear plastic containers for reagents.
- Microtubes - 6 tubes for preparing nitrate standard dilutions
- 5 ml clear tube - (1) for preparing nitrate standard dilution

### Supplied by User

- 25 ml graduated cylinder.
- Pipetters - variable pipettors (10/100 µl and 1 ml) or multi-well pipetter
- Several test tubes
- Vortex-type mixer
- Microplate mixer
- Microplate reader capable of reading at 540 nm +/- 20 nm
- Timer (0 to 20 minutes) - a clock or stop watch is adequate
- Deionized or distilled water (di-water) - Nitrate and Nitrite free!
- 2.5 ml of concentrated Hydrochloric Acid (HCl)
- Small beaker with ice

### NECI The Nitrate Elimination Company, Inc.

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## 2. NITRATE TEST KIT - LOW RANGE (Catalog No. M-NTK-302)

### A. Reagent Preparation

Prepare on day of use.

1. Assay Buffer - ready to use from kit. Warm to room temperature for nitrate tests. If desired, the Assay Buffer may be more quickly warmed in a 30°C water bath.
2. Remove tube of NaR from amber bag and add 1.0 ml Assay Buffer. Mix for 3 seconds with a vortex-type mixer. Allow to stand at room temperature for 20 minutes, with 3 seconds of vortex-mixing at 10 and 20 minutes. Keep on ice until use.
3. Prepare 3 N HCl by adding 2.5 ml of concentrated HCl to 7.5 ml di-water. Mix.
4. Add 10 ml 3N HCl to Color Reagent 1 bottle. Mix by inverting several times.
5. Add 10 ml di-water to Color Reagent 2 bottle. Mix by inverting several times.
6. Remove tube of NADH from amber bag and tap tube to settle contents. Add 0.6 ml di-water and replace cap. Mix for 3 seconds with a vortex-type mixer. Keep on ice until use.
7. Add di-water to the 5 ml line of the clear tube labeled Quench Agent. Mix thoroughly.
8. In pipetter reservoir basin (included in kit), add 9 ml Assay Buffer, 1 ml NaR solution (prepared in Step 2), and 0.5 ml NADH solution (prepared in Step 6). Mix thoroughly by stirring with a pipette tip.

### B. Standard Preparation

Transfer 0.5 ml of 100 ppm nitrate standard (provided in kit) to a test tube. Dilute with 4.5 ml of di-water to make a 10 ppm nitrate standard. In the 5 ml clear tube (provided in kit), dilute 0.5 ml of the 10 ppm standard with 4.5 ml of di-water to make a 1 ppm nitrate standard. Use this 1 ppm standard and the 6 microtubes to prepare nitrate standards as shown in table below.

µl of 1 ppm nitrate standard	µl of di-water	Resulting Standard (ppm)	Resulting Standard (µM)
1000 µl	0 µl	1.0 ppm	71.4 µM
500 µl	500 µl	0.5 ppm	35.7 µM
250 µl	750 µl	0.25 ppm	17.9 µM
125 µl	875 µl	0.125 ppm	8.9 µM
50 µl	950 µl	0.05 ppm	3.6 µM
0 µl	1000 µl	0.0 ppm	0.0 µM

### C. Procedure

The following procedure uses 2 replicates of each standard, unknown sample and reagent blank. Using the Microplate Sample Template, assign 2 wells for each standard, sample and reagent blank.

1. Pipet 50 µl di-water into 2 wells for use as reagent blanks.
2. Pipet 50 µl of each nitrate standard and sample into designated wells.
3. Pipet 50 µl NaR-Assay Buffer-NADH mixture (prepared in Step 8 of Reagent Preparation) to each well. Shake on a plate mixer for ~20 minutes @ 800 rpm.
4. Pipet 30 µl Quench Agent to each well. Shake plate for ~10 min.

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**NOTES ON THE TEST****Unknown Samples with High Nitrate**

This NECl Nitrate Test Kit is capable of determining nitrate levels of up to 1 ppm nitrate-N (71.4 µM). If an unknown sample is found to have more than 1 ppm nitrate-N, the sample may be diluted with di-water 1:10 to allow an exact determination. For example, take 100 µl of sample and add 900 µl of deionized water to make a 1:10 dilution, and then assay 50 µl of the diluted sample. After finding the amount of nitrate in the sample, multiply the nitrate concentration by 10 to find the nitrate concentration in the original sample. **NOTE:** Keep the sample volume constant by diluting the sample rather than using a smaller volume of sample in the assay.

**Testing for Nitrite?**

Nitrite can be determined by replacing the solution in Step 3 of the Procedure with 50 µl of di-water and eliminating Step 4.

**Notes on the Reagents**

- **Assay Buffer** - 25 mM K-PO<sub>4</sub>, 0.025 mM EDTA; pH 7.5
- **Color Reagent 1** - 1% Sulfanilamide in 3N HCl
- **Color Reagent 2** - 0.02% N-Naphthylethylenediamine in di-water
- **NADH** - 1 mM NADH
- **Nitrate Reductase (NaR)** - approx. 0.5 unit of NaR per tube
- **Nitrate Standard** - 1 vial of 100 ppm nitrate-N
- **Quench Agent** - mild, non-toxic oxidizing chemical to remove excess NADH.

**Caution**

Use care when handling samples containing HCl. Gloves are recommended.

**Waste Disposal**

Follow all local guidelines and regulations. If no local guidelines apply to your situation, wash waste down a sink using large amounts of running water.

**Questions?**

Call us at **1-888-NITRATE** (1-888-648-7283)  
or e-mail questions to [tech@nitrate.com](mailto:tech@nitrate.com)  
Visit NECl Web — [www.nitrate.com](http://www.nitrate.com) —  
for answers to frequently asked questions.



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**C. Procedure (cont.)**

5 If using a multi-pipetter, rinse the used multichannel pipetter basins with **di-water**. Transfer **Color Reagent 1** to one basin and **Color Reagent 2** solution to the other basin.

6 Add **50 µl Color Reagent 1** and **50 µl Color Reagent 2** solution to each well.

Shake on a plate mixer for ~10 minutes @ 800 rpm.

7 **Zero the plate reader with the reagent blank wells using a 540 nm filter** (or a filter as close to 540 nm as possible).

8 **Read the absorbances of all wells with standards and samples.** Transfer results to your computer system for analysis and printing.

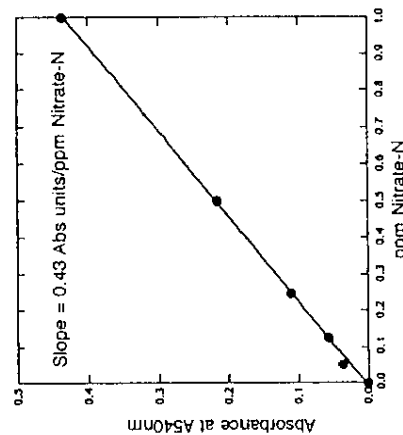
**D. Calculations**

1 To correct for any background absorbance due to the reagents, subtract the mean absorbance of the reagent blanks from the mean absorbance of each nitrate standard and unknown sample:

$$\text{corrected mean sample A-540 nm} = (\text{mean A-540 nm for samples}) - (\text{mean A-540 nm for reagent blanks})$$

2 Generate a standard curve for the nitrate standards (see example below). Using linear graph paper or computer plotting program such as Sigma Plot® or spreadsheet such as Excel®, plot the ppm nitrate-N on the x-axis, and the A-540 nm for each nitrate standard on the y-axis. If plotting by hand, draw a straight line through the points for the nitrate standards. If plotting by computer, the slope of the line can be calculated for determining nitrate-N ppm in the unknown sample.

3 Using the standard curve, determine the ppm nitrate-N for the sample: (a) Find the corrected A-540 nm for the sample on the y-axis of the standard curve. (b) Follow over along a horizontal line to where the line intersects the standard curve. Trace down to the x-axis and read the ppm of nitrate-N on the x-axis.



Typical nitrate standard curve generated with NECl Nitrate Test Kit.

Note: 1 ppm nitrate-N = 71.4 µM nitrate. Slope = 0.006 A-540 nm per µM Nitrate.